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(71) Applicant *(for all designated States except US)*: SMITH & NEPHEW PLC [GB/GB]; 2 Temple Place, Victoria Embankment, London WC2R 3BP (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WOLOWACZ, Richard [GB/GB]; 16 Station Road, Tadcaster, Yorkshire LS24 9JG (GB). WOLOWACZ, Sorrel [GB/GB]; 16 Station Road, Tadcaster, Yorkshire LS24 9JG (GB). SHERIDAN, Julie, Marie [GB/GB]; 25 Wilkinson Way, Strensall, York YO3 25ZA (GB).

(74) Agent: SMITH & NEPHEW GROUP RESEARCH CENTRE; Group Patents & Trade Marks, York Science Park, Heslington, York YO10 5DF (GB).

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(57) Abstract

There is disclosed the use of matrix metalloproteinase (MMP) inhibitors e.g. collagenase, stromeylsin, gelatinase inhibitors in the production of tissue equivalents. The inhibitors are used in particular to inhibit MMPs present in animal serum used in the production technique, thereby increasing collagen deposition. Tissue culture media and extracted animal serum containing a supplemented MMP inhibitor are also disclosed.

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METHODS FOR THE PREPARATION OF ARTICICIAL CELLULAR TISSUE

The present invention relates to the field of cell culturing and in particular relates to improved methods for culturing cells in vitro to form a tissue equivalent (i.e. tissue engineering) for the treatment of tissue defects in mammalian patients.

The treatment of tissue defects such as skin ulceration in various forms e.g. pressure sores and venous ulcers, serious burns and other tissue defects such as articular and meniscal cartilage defects, snapped ligaments, tendons and the like has in recent times being directed towards the production, in vitro, of a tissue equivalent which is then grafted onto the defect site to replace or augment the damaged tissue. Typically this in vitro production involves first obtaining a sample of autologous or allogeneic cells. Where autologous cells are utilised these are generally harvested from an unaffected site on the patient. Where allogenic cells are utilised these may be obtained from a variety of different sources such as tissue banks. Cells extracted from the sample are usually cultured in an aqueous medium where they proliferate and are culture-expanded by passage. The media is usually supplemented with an animal serum to provide some of the necessary constituents for optimal cell growth and proliferation.

The cells from the culture expansion are then usually seeded onto a supporting structure such as a bioresorbable three dimensional matrix or seeded into a supporting structure such as collagen gel to form a seeded construct and then, in the presence of an aqueous serum-containing media, the construct is incubated for a sufficient period of time to form either an equivalent to the tissue to be replaced or is developed in vitro to the point where it is capable

of being grafted to the defect site and develop further into such a tissue equivalent.

This in vitro process can take some time which serves to increase patient discomfort and hospital costs.

It is therefore an object of the present invention to provide improved methods for the in vitro culturing of cells and in particular, improved methods for the production of tissue equivalents.

The formation of an integral tissue requires the production of an extracellular matrix (ECM), an intricate network of macromolecules that not only binds cells and tissues together but also influences the development, polarity and behaviour of the cells it contacts. A major constituent of the ECM are collagens, a family of highly characteristic fibrous proteins. Collagen is present in a variety of known types with particular tissues displaying a predominance of a particular form. For example, cartilage is associated with collagen II and collagen IX. Skin and tendon is associated with collagen I and III. Bone and meniscus cartilage is associated with collagen I. Other constituents of the ECM include glycosaminoglycans, fibronectin, vitronectin and other growth factor binding proteins e.g. latent transforming growth factor binding proteins (LTBP) 1,2,3. The constituents of the ECM are secreted locally by cells in the ECM. In most connective tissues these constituents are secreted largely by fibroblasts. In some specialised connective tissues, such as cartilage and bone, they are secreted by cells such as chrondrocytes and osteoblasts respectively.

The present invention is based, in part, on the premise that certain proteins present particularly in serum containing media

commonly used in cell culturing and commercially available as such may in fact have an inhibitory effect on the formation of ECM components during the in vitro production of tissue equivalents. That is, the presence of enzymes especially of the gelatinolytic type which are present in the serum degrade some of the collagen and other ECM components produced by the culturing cells. As a result, the formation of an ECM is prolonged and consequentially so is the formation of an engraftable tissue equivalent.

In accordance with the presently disclosed invention, there is provided a method for the in vitro production of a tissue equivalent, said equivalent comprising mammalian cells supported by a scaffold, which method comprises the step of containing said cells supported by a scaffold in the presence of an effective amount of a matrix metalloproteinase inhibitor.

In another aspect of the present invention, there is provided a tissue culture media comprising an effective amount of a matrix metalloproteinase inhibitor.

In another aspect of the present invention, there is provided the use of a tissue culture media comprising an effective amount of a matrix metalloproteinase inhibitor.

In a further aspect of the present invention, there is provided a method for the treatment of tissue defects on a mammalian patient, said method comprising the step of applying to said patient a tissue equivalent as provided hereinbefore.

In yet a further aspect of the present invention, there is provided a method for reducing matrix metalloproteinase activity in

tissue culture media, said method comprising the step of admixing said tissue culture media with an effective amount of a matrix metalloproteinase inhibitor.

In another aspect of the present invention, there is provided a method for increasing the rate of collagen deposition during the in vitro production of a tissue equivalent, said equivalent comprising mammalian cells supported by a scaffold, wherein said method comprises the step of containing said cells supported by a scaffold in the presence of an effective amount of a matrix metalloproteinase inhibitor.

The term "matrix metalloproteinase inhibitor" is intended to mean an inhibitor i.e. a substance that is capable of restricting, hindering or preventing the activity of a matrix metalloproteinase (MMP).

The amount of collagen deposition during the production of a tissue equivalent is the equilibrated result of collagen production by the culturing cells and the rate of collagen degradation mainly by MMP's present in the serum containing tissue culture media. The present invention pushes this equilibration in favour of overall collagen deposition by inhibiting collagen degradation as a result of serum MMP activity.

Matrix metalloproteinases are naturally occurring proteases present in the mammalian body which are believed to be involved in a number of biological processes which include remodelling of extracellular matrix components, angiogenesis and metastatic invasion of tumour cells. A number of MMPs have now been identified although this may not necessarily be exhaustive of all

MMPs present in the mammalian body. Nagase et al : (1992) Matrix, supplement No 1, pp421-424, incorporated herein by reference, proposed a number of criteria for classifying a new proteinase as an MMP. These criteria include; a proteinase whose activity is blocked by 1,10 phenanthroline ; which exists in a latent form activated by organomercurial compounds; which is inhibited by tissue inhibitor of matrix metalloproteinase (TIMP), which hydrolyses at least one ECM component; requires calcium for activity/stability; a proteinase which has zinc as an intrinsic metal ion. A particularly important criteria proposed is gene homology to collagenase (MMP1). For a review of the classification system currently used for MMPs, see Sang OA et al; J.Protein Chem; (1996); 15(2), pages 137 to 160 and Cuvelier A et al; Rev-Mal Respir; (1997); 14(1); pages 1-10, both of which are incorporated herein by reference.

MMPs may be conveniently divided into four classes, collagenases, gelatinases, stromelysins and more recently membrane type MMP (MT-MMP). Within each class, there are individual MMP types (herein called 'members'). A particular class of MMP or even a particular MMP member may be specifically inhibited in the presence of an MMP inhibitor although frequently an inhibitor is not specific for a particular class or member but rather displays selectivity towards inhibiting a particular class or member.

It is currently thought that the class of collagenases includes at least the members MMP 1, 8 and 13. Collagenases are a class of MMP believed to be primarily responsible for the in vivo cleavage of native triple helical fibril collagen. For a review of currently known fibrillar collagens see the monograph Kadler K; (1994) Protein profile, extracellular matrix 1: fibril-forming collagens; Academic Press, London, incorporated herein by reference. Collagenase

inhibitors may be either synthetic or natural. Natural collagenase inhibitors include tissue inhibitors of matrix metalloproteinase (TIMPs) 1,2,3 and 4. For a review of the current classification of TIMP proteins see FEBS-Lett (1997) 20; 401(2-3); 213-7, incorporated herein by reference. Examples of known collagenase inhibitors are disclosed in WO95/24921, incorporated herein by reference, which refers to a number of collagenase inhibitors. Collagenase inhibitors include inhibitors based on hydroxamic acid, see WO 90/05716, WO90/05719 and WO92/13831, all of which are incorporated herein by reference. Other zinc binding inhibitors include derivatives of formylhydroxylamine, sulphydryl, phosphinate and carboxylates. An example of a known hydroxamic acid inhibitor is known by the trade name GALARDIN, a synthesis for which is disclosed in US 5189178 and US 5114953, both of which are incorporated herein by reference. For further collagenase inhibitors see also EP-A-126,974, EP-159396, US 4599361 and US 4743587, all of which are incorporated herein by reference. Further examples of collagenase inhibitors include collagenase antibodies, either polyclonal or monoclonal and includes the Fab fragments thereof. Other examples of collagenase inhibitors include EDTA, cysteine and tetracyclines.

At present, it is believed that the class of stromelysins includes at least the members MMP 3 and 6. These MMPs have a wide range of ECM substrate specificity which includes laminin and glycosaminoglycans (GAGs). Examples of known stromelysin inhibitors include substances currently under development for the treatment of cancer and known by the designations, CDP-845 (Celltech), CH715 (Chiroscience), GALARDIN (Ligand pharmaceutical) and L-758354 (Merck). Polyclonal or monoclonal

antibodies including Fab fragments to the stromelysin class or members thereof may also be utilised.

Currently it is thought that the class of gelatinases includes at least the members MMP 2 and 9. Gelatinases display a substrate specificity towards basement collagen and denatured fibrillar collagen. Examples of known gelatinase inhibitors include the tetracyclines, chemically modified tetracyclines (CMTs), CDP-845, CH-715, CT-1746 and Rega 3G12. Useful CMT's include doxycycline and minocycline and preferred CMT's include CMT 2,3,7,8. CMT 6 may also be used but is less favoured due to poor solubility in aqueous media in the absence of a further solvent e.g. Dimethyl sulfoxide (DMSO). It is understood that antibodies and fragments thereof to the gelatinase class or members thereof may be utilised.

The inhibitor of the present invention may be irreversible, or reversible in which case they may be of the competitive, non-competitive, uncompetitive or mixed type. The type of inhibition displayed can be determined enzymologically according to standard textbook protocol. See, for example, Cornish-Bowden ; Fundamentals of enzyme kinetics; Butterworths, London. The inhibitor is preferably water soluble and furthermore does not adversely effect cell viability or phenotype expression. The inhibitor such as those illustrated above may be selective for a particular class of MMP or even a particular member. Inhibitors selective for the gelatinase class or particular members thereof are preferred selective inhibitors although MMP inhibitors that are non-selective i.e. inhibit a broad range of MMP's in more than one class are particularly preferred in the present invention. Apt examples of non-selective MMP inhibitors include the tetracyclines and the CMTs

1,2,3,6,7 and 8 (available from CollaGenex, Newtown, PA USA). Particularly preferred non-selective tetracycline MMP inhibitors include doxycycline and the specific compound tetracycline, oxytetracycline and sancycline (available from Sigma Co.)

It will be understood by those skilled in the art that inhibition of a broad range of MMPs may be achieved by providing a media with a range of selective MMP inhibitors.

An effective amount of the inhibitor should be used. By the term "effective amount" we mean an amount of inhibitor sufficient to produce a detectable reduction in MMP activity in serum-containing tissue culture media when compared with appropriate control e.g. in tissue culture media that has not been supplemented with a MMP inhibitor.

It is preferred however, that MMP activity is reduced by at least 10%, favourably by at least 30%, more preferably by at least 50%, even more preferably by at least 70% but most preferably by at least 80% when compared with appropriate control. MMP activity in the tissue culture media of the present invention may be measured by fluorescence substrate analysis, see Knight CG et al (1992); Biochem J; 260, p259-263, incorporated herein by reference. Model substrates for collagenase, gelatinase and stromelysin classes which may be measured by fluorescence include fluorescence groups 7-methoxycoumarin and N-methylanthranilic acid attached to a short peptide.

Inhibition of this activity is indicative of a MMP inhibitor.

The tissue culture media of the present invention may be prepared from commercially available basal media supplemented with animal serum, antibiotics such as penicillin and streptomycin, glutamine, buffers such as Hepes, non-essential amino acids, other factors and further supplemented with the inhibitor. Suitable basal media includes Ames medium, Dulbeco's modified Eagles medium (DMEM), Basal medium Eagle (BME), BGJB medium, Roswell Park Memorial Institute media (RPMI), Click's medium, CRCM-30 medium, CMRL-1066 medium, Minimally Essential medium (MEM), (available from Sigma Biosciences).

Animal serum typically utilised in current cell culturing techniques include bovine, ovine, equine, human, chicken, goat, porcine and rabbit derived serum. Animal serum may be derived from fetal or neo-natal animals. Commonly, serum is derived from foetal animals. Preferably, the serum of the present invention is ovine or bovine. A common and popular animal serum in current cell culturing techniques is foetal calf serum. Preferably, the animal serum utilised in the present invention is foetal calf serum at a concentration of between 5 to 60% v/v, more preferably 10% to 20% v/v.

It should be understood that the present invention may also find utility with serum-free media (so-called 'defined media') since mammalian cells themselves secrete a basal level of MMP during normal cellular activity.

The inhibitor may be admixed with the media prior to cell culturing although it may be added at any appropriate time during the culturing process. Indeed it may be desirable to admix after the

culturing process has begun and in particular after the cells have been allowed to proliferate for several days.

Cells used in the present invention are typically mammalian autologous or allogeneic cells. Xenogeneic cells may be used but are currently unfavourable due to immunological problems with their use. The present invention is useful for any cell culturing process where the formation of an ECM is required but is particularly suited to the production of a tissue equivalent. Autologous cells may be obtained by biopsy from the patient and treated with degradative enzymes e.g. trypsin and collagenases to separate the cells for use in the culturing process. Allogenic cells may be obtained from a number of sources such as tissue banks.

The cells used in the present invention may be terminally differentiated or capable of undergoing phenotypic change e.g. stem cells, committed cells, pluripotent stem cells and other progenitor cells. It is understood that this includes the processes of differentiation, de-differentiation and trans-differentiation. Cells useful therefore include mesenchymal derived cells, epithelial and endothelial cells. Cells useful in the present invention include fibroblasts, keratinocytes, chrondrocytes, tenocytes, ligamentocytes, fibrochrondrocytes, bone marrow stromal cells and other mesenchymal stem cells e.g. dermal papillae cells. Other cells include melanocytes, hepatocytes, pancreatic and urothelial cells.

The invention is particularly useful in the production of loose, dense, cartilagenous and mineralized connective tissue equivalents. Suitable examples thereof include skin, meniscus and articular cartilage, tendons, ligament and bone.

By the term 'tissue equivalent' we mean an *in vitro* produced structure comprising cells supported by an artificial scaffold, whereby said structure emulates the structural and functional characteristics of a particular tissue either when grafted to or following grafting to a target site on a patient.

illustrative examples of tissue equivalents available currently and whose production may benefit from the method of the present invention include tissue equivalents such as DERMAGRAFT (Trademark) and APPLIGRAF™. The present invention is further useful in the production of cell seeded wound dressings, see for example, our patent applications WO 91/13638 and WO 97/06835 both of which are incorporated herein by reference.

Further methods for the manufacture of tissue equivalents are disclosed in, for example, US 5,460,939, WO90/02796, WO 90/12603, US 5,256,418, WO96/13974, all of which are incorporated herein by reference.

Suitable scaffold materials are preferably biodegradable and are not inhibitory to cell growth or proliferation. Preferably the materials should not elicit an adverse reaction from the patients body and should be capable of sterilisation by e.g. ethylene oxide treatment (allowing sufficient time for degassing), prior to seeding with cells . Suitable materials therefore include biodegradable polyesters such as polylactic acid (PLA), polyglycolic acid (PGA), polydioxanone, polyhydroxyalkanoates e.g. polyhydroxybutyrate (ICI) and hyaluronic acid derivatives e.g. HYAFF (Fidia). Further suitable materials include those disclosed in our patent applications WO 91/13638 and WO 97/06835, incorporated herein by reference such as hydrophilic polyurethanes, polyetherpolyester, polyethylene

oxide, polyetherpolyamide, carboxymethylcellulose, ethylene-vinyl acetate copolymers, polybutadienes, styrene-butadiene-styrene block copolymers and the like.

Other scaffold materials are collagen based e.g. cross-linked collagen/elastin material, cross-linked collagens manufactured from acid-soluble type I bovine collagen sources, collagen gels, COLLASTAT (trade name, Vitaphore), COLETICA (trade name). Collagen from natural or recombinant sources may be used.

The scaffold of the present invention may be in the form of a three dimensional matrix or a layer, for example a continuous film or gel. The matrix structure may be manufactured from fibres of a suitable material which is then textile processed (e.g. braided, knitted, woven or non-woven, melt-blown, felted and hydroentangled) and further manipulated into a desired three dimensional shape e.g. ligament or tendon following which the matrix is seeded with cells which are optionally predispersed in collagen or fibrin gel. The matrix structure may also assume other forms e.g. sponges or foams.

The in vitro process generally comprises submerging the cells or structure comprising cells supported by a scaffold, in the tissue culture medium of the present invention contained within a cell culture vessel such as a cell culture flask, said flask formed from suitable materials such as high impact polystyrene and which is preferably transparent to allow viewing of the culturing process. The cell culture is then incubated at physiologically acceptable conditions over several days until the desired state is achieved. Optionally, during the culturing process a proportion of the tissue culture

medium of the present invention is replaced with fresh medium (so called 'split feeding').

In accordance with a further aspect of the present invention there is provided a tissue culture media comprising an artificial i.e. supplemented matrix metalloproteinase inhibitor.

In accordance with a further aspect of the present invention there is provided extracted animal serum in a form suitable for cell culturing techniques comprising an artificial matrix metalloproteinase inhibitor.

In accordance with a further aspect of the present invention there is provided a method of producing a tissue equivalent comprising cells supported by a scaffold, said method comprising the step of containing said cells supported by a scaffold in tissue culture media comprising animal serum, which media is supplemented with a matrix metalloproteinase inhibitor.

The present invention will now be illustrated by way of example.

Examples 1 to 10: Manufacture of tissue equivalents

Example 1

Preparation of tissue scaffold for cell seeding

Braided polyester three dimensional tubular scaffolds (approx. 40mm length by 3mm) were soaked in 70% v/v alcohol in water for 1.5 hours and left to soak for 24 hours in antibiotic/antimycotic solution at 4°c. The scaffolds were removed from antibiotic wash and rinsed in sterile distilled water, sterile phosphate buffered

solution (PBS) and in tissue culture medium (TCM) comprising 10% fetal calf serum (consisting of : DMEM (with sodium pyruvate, 1000mg/L glucose, pyridoxine) 85% v/v, Gibco Ltd), 10% v/v heat inactivated foetal calf serum (Gibco Ltd), 0.02% v/v Hepes buffer (1M, Gibco Ltd), 0.01% v/v non essential amino acids; 0.01% v/v penicillin/streptomycin (5000IU/ml - 5000µg/ml) and 0.01% v/v L-glutamine). Scaffolds were placed in fresh TCM and incubated at 37°c.

Seeding and culturing of tissue scaffold

Samples of human foetal foreskin fibroblasts (Huffs) were suspended in TCM further supplemented with absorbate (72µg/ml) to form a cell suspension and cell counted using a modified Fuchs Rosenthal haemcytometer. Cell density of approx. 5.8 $x10^6$ cells were seeded per scaffold, each scaffold had a volume of approx. 212mm^3. Scaffolds were then placed into centrifuge tubes with cell suspension (5ml), sealed and placed on a shaker to agitate at 37°c overnight. Seeded scaffolds were then removed from the shaker and placed three seeded scaffolds (herein 'devices') per culture flask into culture flasks (Falcon T25 flasks, Becton Dickinson) containing TCM (10ml). The flasks were then transferred to CO_2 incubators and incubated at 37°c.

After 24 hours, several devices were removed and one was visually examined for cell attachment using ethidium/calcein stain (Live/dead stain, Eukolight™, Cambridge Biosciences). The remaining removed devices were analysed for collagen using a hydroxyproline assay based on Kivirikko et al ; (1967); Anal.Biochem., 19; p249 to 255 and for histological examination. The remaining devices were, after a further 24 hours, split fed by removing 5ml TCM and replacing with 5ml fresh TCM

(supplemented with 72μg/ml ascorbate). In addition, one third of the remaining cultures were supplemented with 20μg/ml doxycycline (CollaGenex, Inc., Newtown, PA, USA) (and were designated group 2), one third with 40μg/ml doxycycline (group 3), with the remaining third containing no doxycycline (group 1). Cultures were then incubated for 12 days, split feeding on alternate days, supplementing with fresh doxycycline where applicable.

Four weeks following initial seeding, six devices from each group were taken for histological and biochemical evaluation for total DNA, total collagen and total glycosaminoglycan (GAG). The results of the analysis for total collagen from the four week study are displayed in Table 1.

Table 1: Total average collagen (hydroxyproline assay) per device according to example 1 (four weeks)

Device Group	Total collagen (μg/device) ± S.D
1	3.0 ± 3.0
2	34.6 ± 6.0
3	30.2 ± 4.3

Example 2

The method of claim 1 was repeated at the 40μg/ml level only using PLA yarns in place of polyester braids. The results are displayed in table 2.

Table 2: Total average collagen (hydroxyproline assay) per device (four weeks) according to example 2

Device group	Total Collagen (μg/device)
Group 1	22.375 ± 15.96
Group 3	134.0 ± 12.15

Example 3

Ovine articular chrondrocytes were isolated from 8 week old lambs by digestion in 0.2% collagenase at 37°c. The isolated cells were seeded onto PGA felts and cultured in DMEM supplemented with FCS (10%) in Apollo bioreactors (ATS, La Jolla, CA) at a seed level of 4×10^6 cells per bioreactor either without supplemented doxycycline (control, group 1) or with supplemented doxycycline (40μg/ml, group 3). Collagen levels measured after four weeks are represented in table 3 below.

Table 3: Total average collagen (hydroxyproline assay) per device according to example 3

Device group	*Total collagen (% collagen of dry weight)*
1	2.4
3	4.7

Example 4

The method of example 1 was repeated using PLA yarns, Huff fibroblasts and the following inhibitors:

GALARDIN (10 nmolar, 100 nmolar)

Peptide hydroxymate N-1405 (Bachem, 10μmolar and 1μmolar)

minocycline (100μg/ml, Sigma)

Tetracycline (200μg/ml, Sigma)

Sancycline (150μg/ml)

TIMP 1,2,3 and 4 (10^{-11}molar, Celltech).

Increased collagen content was observed in the test samples compared to control.

Example 5

The method of example 4 was repeated using PGA felts.

Increased collagen content was observed in the test samples compared to control.

Example 6

The method of example 4 and 5 was repeated using ovine meniscal chrondrocyte

Increased collagen content was observed in the test samples compared to control.

Example 7

The method of example 4 and 5 was repeated using keratinocytes (Human SCABER cells, American Type culture collection , Maryland, USA).

Increased collagen content was observed in the test samples compared to control.

Example 8

The method of example 4 and 5 was repeated using tenocytes (human, surgical discard)

Increased collagen content was observed in the test samples compared to control.

Example 9

The method of example 4 and 5 was repeated using human mesenchymal stem cells derived from bone marrow.

Increased collagen content was observed in the test samples compared to control.

Example 10

The method of claim 4 and 5 was repeated using endothelial cells derived from human umbilical sources.

Increased collagen content was observed in the test samples compared to control.

Example 11: Investigation of MMP activity in commerically available animal sera

Xcell II zymography system (Novex) was assembled according to the manufacturer's instructions with 2 Novex precast gelatin gels. Test sera samples were activated using aminophenylmercuric acetate (APMA, 10%). As a control, sera samples were not activated with APMA. Sample buffer (Tris-HCL (0.5M, 400µl, pH 6.8), Sucrose (60%, 2.5ml), SDS (20%w/v, 1.75ml), H_2O (350µl), Bromophenol Blue, (1mg) was mixed 1:1 with the sample sera (obtained from Gibco) below and loaded onto both gels.

Well Content	Well number
Foetal Calf Serum (1:5 dilution, activated)	1
Foetal Calf Serum (1:5 dilution,unactivated)	2

Calf Serum (1:5 dilution, activated)	3
Calf Serum (1:5 dilution, unactivated)	4
Recombinant MMP2 (10ng/µl)	5
Recombinant MMP9 (10ng/µl)	6

Recombinant MMP2 and 9 were obtained from Chemicon.

The gels were allowed to run at 125v, 40mA for 90 minutes, following which the gels were disassembled and placed in renaturing buffer according to manufacturers instructions for 30 minutes. The gels were then transferred into developing buffer for overnight incubation at 37°c. The gels were then removed from the incubator and placed into 10% methanol, 10% acetic acid (destain solution) for 20 minutes agitation. The gels were then transferred into Coomassia Blue-R-250 stain for 20 minutes agitation and then back into destain for 15 minutes. The destain solution was changed and then agitated for 20 minutes before being changed again for a final 30 minutes agitation. The results are displayed in fig.1.

Results

Well 6 illustrates MMP 9 control used as a marker. Two bands are evident, one being the active form of MMP 9 and one being the proform. Well 5 illustrates MMP2 band as a marker. Well 1 displays prominent bands corresponding to the active and proform of MMP9 with lighter but evident MMP 2 activity. Well 2 displays prominent levels of MMP 9 proform and lighter but still evident proform MMP 2 activity. Wells 3 and 4 displays evident MMP 9 activity which is less than that observed with wells 2 and 3. Light but evident bands of MMP 2 pro and active forms were present in wells 3 and 4. These results indicate the present of MMP activity in commercially available animal sera.

CLAIMS

1. A method for the in vitro production of a tissue equivalent, said equivalent comprising mammalian cells supported by a scaffold, which method comprises the step of containing said cells supported by a scaffold in the presence of an effective amount of a matrix metalloproteinase inhibitor.

2. The method of claim 1 wherein the inhibitor is a non-selective inhibitor.

3. The method of claim 2 wherein the non-selective inhibitor is a tetracycline or a chemically modified tetracycline.

4. The method of claim 3 wherein non-selective inhibitor is doxycycline, sancycline or minocycline.

5. The method of claim 1 wherein the inhibitor is selective for a collagenase.

6. The method of claim 5 wherein the collagenase selective inhibitor is a TIMP or a hydroxamic acid based inhibitor.

7. The method of claim 1 wherein the inhibitor is selective for a stromelysin.

8. The method of a claim 1 wherein the inhibitor is selective for a gelatinase.

9. The method of claim 8 wherein the gelatinase selective inhibitor is a tetracycline or chemically modified tetracycline.

10. The method of any preceding claim wherein the cells supported by a scaffold are contained in tissue culture media comprising animal serum.

11. The method of claim 10 wherein the animal serum is bovine, ovine, equine, human, chicken, goat, porcine or rabbit derived.

12. The method of claim 11 wherein the serum is foetal calf serum.

13. The method of any preceding claim wherein the cells are terminally differentiated or capable of undergoing phenotypic change.

14. The method of claim 13 wherein the cells are mesenchymal derived, epithelial or endothelial cells.

15. The method of claim 14 wherein the cells are fibroblasts, keratinocytes, chrondrocytes, tenocytes, ligamentocytes or fibrochrondrocytes.

16. The method of any preceding claim wherein the equivalent is a skin, meniscal cartilage, articular cartilage, tendon, ligament or bone tissue equivalent.

17. The method of any preceding claim wherein the scaffold is a three dimensional matrix.

18. The method of claim 17 wherein the matrix comprises polylactic acid, polyglycolic acid or copolymers thereof.

19. A method of reducing matrix metalloproteinase activity in a tissue culture media comprising the step of admixing the media with an effective amount of matrix metalloproteinase inhibitor.

20. A tissue culture media comprising a matrix metalloproteinase inhibitor.

21. The media of claim 21 further comprising animal serum.

22. Extracted animal serum in a form suitable for use in cell culturing techniques further comprising an artificial matrix metalloproteinase inhibitor.

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INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/GB 98/02147

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/06 C07K14/81

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 550 760 A (FUJI YAKUHIN KOGYO KK) 14 July 1993 see abstract see page 3, line 5 - line 25 see page 7, line 22 - line 41 see table 3 --- -/--	19,20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 November 1998	16/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Panzica, G

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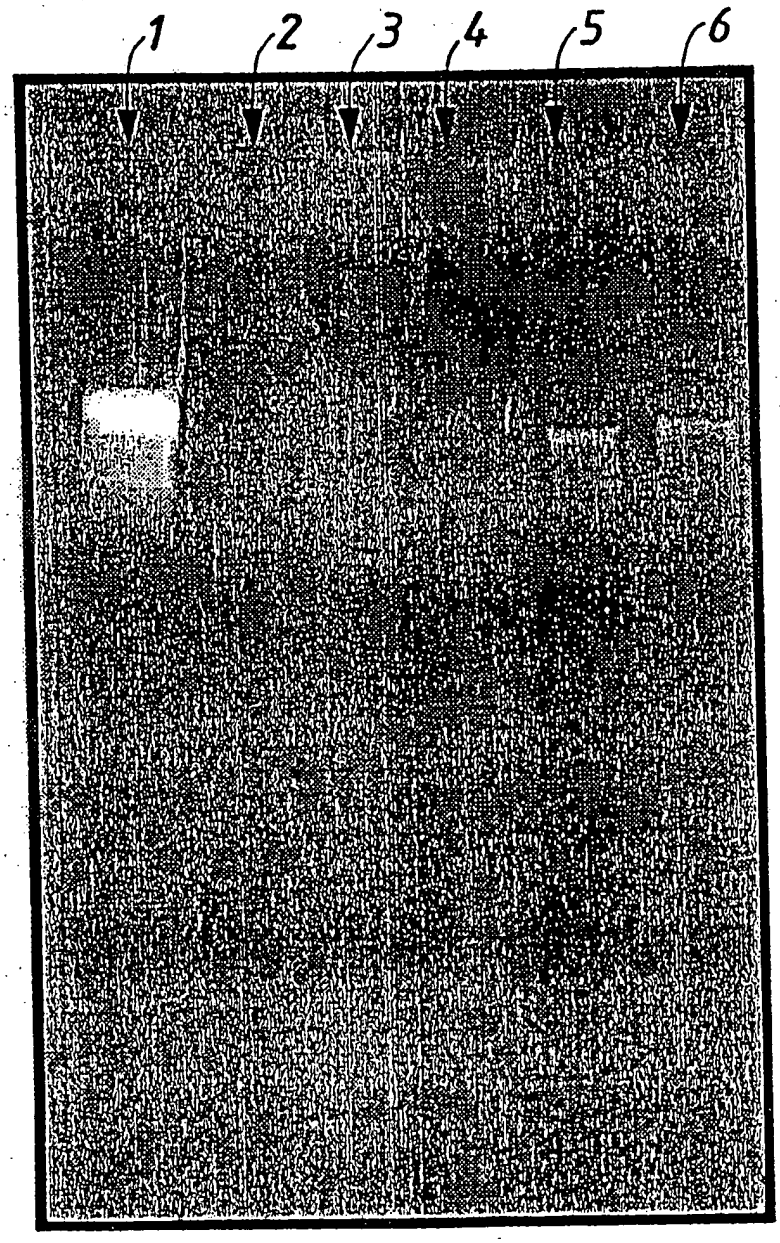
FIG.1.



INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/GB 98/02147

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

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Int. ional Application No
PCT/GB 98/02147

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